Naval Medical Research Institute
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MELATONIN DOES NOT PROVIDE PROTECTION AGAINST HYPERBARIC OXYGEN (HBO) INDUCED SEIZURES

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TECHNICAL REVIEW AND APPROVAL
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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council.

This technical report has been reviewed by the NMRC scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

RICHARD B. OBERST
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Commanding Officer
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14. ABSTRACT

Acute exposure to hyperbaric oxygen (HBO) can result in toxicity to the central nervous system (CNS). The most onerous manifestation of CNS oxygen toxicity is the onset of generalized tonic-clonic seizures. HBO-induced convulsions are of particular importance to the Navy, as it is a limiting factor in the duration of diving missions involving surface supplied oxygen and use of the LAR V and MK-15/16 closed-circuit SCUBA apparatus, and the application of HBO medical treatment for decompression sickness. The causal mechanism of HBO-induced seizures is presently unknown. Melatonin has not been tested in its capacity as an anticonvulsant in HBO conditions likely to produce seizures (6 atmospheres absolute). The goal of this research was to examine melatonin prophylaxis in HBO conditions that reliably produce CNS toxicity.

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INTRODUCTION

Acute exposure to hyperbaric oxygen (HBO) can result in toxicity to the central nervous system (CNS)(1). The most onerous manifestation of CNS oxygen toxicity is the onset of generalized tonic-clonic seizures. HBO-induced convulsions are of particular importance to the Navy, as it is a limiting factor in the duration of diving missions involving surface supplied oxygen and use of the LAR V and MK-15/16 closed-circuit SCUBA apparatus, and the application of HBO medical treatment for decompression sickness (2).

The causal mechanism of HBO-induced seizures is presently unknown. CNS dysfunction as a result of increased exposure to HBO might be related to the deleterious effects of elevated oxygen free radical production (1). The primary utilization of O_2 in the central nervous system occurs during the oxidation of carbohydrates involved in the generation of adenosine triphosphate. Residual O_2 in the brain is reduced to reactive oxygen species assumed to be neutralized by endogenous antioxidative compounds (e.g., glutathione system, superoxide dismutase, and catalase). Endogenous defense mechanisms may be overwhelmed in a HBO environment where the production of oxygen free radicals exceeds normal levels (3-6).

The pineal hormone, melatonin (N-acetyl-5-methoxy-tryptamine) is easily taken up in the brain and is considered to be a potent free radical scavenger (4, 7-12). Melatonin has also exhibited anticonvulsive properties in various seizure models. The severity of pentylenetetrazol-induced convulsions was reduced in gerbils receiving daily subcutaneous doses of melatonin (13, 14). Incidence of potassium cyanide-induced convulsions was reduced in mice given subcutaneous melatonin (12). Kainate-induced convulsions were reduced in rats given intraperitoneal melatonin (15). The occurrence of iron-induced epileptic EEG discharges was reduced in rats given intraperitoneal melatonin (16). Dose-dependent anticonvulsive effects of

melatonin were observed in convulsions induced by intracerebroventricular administration of quinolinic acid, kainate, glutamate, NMDA, and pentylenetetrazole (17). However, in most of the aforementioned seizure models, melatonin did not eradicate convulsions. Lapin, et al. (17) reported longer seizure latencies or suppression of seizures as a function of melatonin administration.

Melatonin has not been tested in its capacity as an anticonvulsant in HBO conditions likely to produce seizures (6 atmospheres absolute (ATA)). The goal of this research was to examine melatonin prophylaxis in HBO conditions that reliably produce CNS toxicity.

EXPERIMENT 1

The rationale for using 6 ATA as a dive depth for these experiments was to minimize time to seizure, minimize the onset of pulmonary toxicity as a complicating factor, and to see if extreme dive profiles are affected by interventions.

Melatonin is more prominently known for its involvement in circadian rhythms and sleep (18). These potential "side-effects" could be equally as harmful in a combat diver scenario, thus we initially sought to test a low dose that might produce prophylaxis in our HBO seizure model. Pablos, et al. (4), reported a reduction in oxidative stress and elevated production of endogenous antioxidant defenses in brain tissues of rats given 10 mg/kg intraperitoneal melatonin prior to 90 minutes of 4 ATA HBO exposure.

We designed our experiment based upon the assumption that the mean seizure latency in control conditions would be approximately 20 min with a standard deviation of 15 min. We sought to detect a difference between control and treatment conditions when the mean seizure latency of the treatment (i.e., melatonin) was at least 45 min. The rationale for seeking a

treatment effect of this size was that it would be operationally important and, assuming depth dependency of any intervention, this time would increase for shallower dives. Our parameters required relatively small sample sizes ($n \geq 7$) to produce power estimates $\geq .80$ when alpha was set at .05 (19, 20).

METHODS

Animals and Injectants

Twenty-four, male Sprague-Dawley rats (mean = 330 g, SD = 39.96 g, Charles River) were randomly assigned to one of three experimental conditions; saline (control), 10% dimethylsulfoxide (Sigma D-8779) in saline (DMSO+saline), or 10 mg/kg melatonin (Sigma M-5250) + 10% dimethylsulfoxide-saline (MEL10). Solutions were prepared a few minutes prior to intraperitoneal administration, immediately before HBO exposure.

HBO Exposure

Animals were individually exposed to HBO. The animal was housed in a 28 x 18 x 13 cm Plexiglas cage with air holes and immediately sealed inside a small hyperbaric chamber (approximately 167 liters). After a 90 s flushing period, 100% oxygen was introduced to the chamber at a rate equivalent to 1 foot of sea water (fsw)/s until reaching 165 fsw (6 ATA). Pressure was maintained at 165±1 fsw for behavioral observation until positive identification of a seizure or pulmonary distress, then the chamber was depressurized at 1 fsw/s and the animal was euthanized.

Behavior Observation

A video camera mounted against a porthole of the hyperbaric chamber provided a clear view of the Plexiglas cage via an external monitor. The primary objective of behavior observation was to identify seizure onset time. The following hallmarks were noted throughout

the dive: facial clonus, forelimb clonus, tonic hindlimb extension, generalized tonic-clonic seizure, and running fits. Pulmonary distress (dypsnea, gagging) was identified and used as an ethical guide to terminate HBO exposure. Animals were observed at all times while in the chamber. The seizure duration of $\geq$ 20 s was selected to ensure positive identification of seizure activity. A list of behavior codes was used as a guide to identify all animal activity during observation (Appendix A). Two dedicated observers verified each other's behavior identification in the animal, and each dive was recorded on videotape for further verification of seizure onset times.

RESULTS

The difference between onset time of a seizure and the time at which the rat reached 6 ATA was calculated to measure seizure latency to the nearest minute. No animals were surfaced for pulmonary reasons. Mean seizure latencies were calculated for each treatment; control (25.0, SD 15.7 min), DMSO-saline (20.4, SD 14.6 min), and MEL10 (25.4, SD 10.0 min). Statistical analysis revealed no significant differences among treatment pairs (DMSO+saline vs. control, $t(14) = -.61, p > .05$; MEL10 vs. control, $t(14) = .06, p > .05$; MEL10 vs. DMSO+saline, $t(14) = -.80, p > .05$).

EXPERIMENT 2

Although 10mg/kg of melatonin reduced oxidative stress and produced a significant increase in brain tissue antioxidants after 90 min of HBO exposure at 4 ATA (4), this dose may not have been sufficient to produce a protective effect in the current CNS toxicity model. Using a quinolinate seizure model in mice, Lapin, et al., (17) observed significantly longer seizure latencies with 25, 50 and 100 mg/kg intraperitoneal administration of melatonin compared to the

control vehicle. Thus we tested higher doses of melatonin in our HBO model, one that represented the higher range (75 mg/kg), and a second dose beyond the range (150 mg/kg), employed by Lapin, et al. (17). Given that we observed no differences in seizure latency between the control and DMSO+saline conditions in Experiment 1, we only used DMSO+saline as the control in Experiment 2.

METHODS

Animals and Injectants

Thirty, male Sprague-Dawley rats (mean = 350 g, SD = 21.84 g, Charles River) were randomly assigned to one of three experimental conditions; 10% dimethylsulfoxide in saline (DMSO+saline), 75 mg/kg melatonin + 10% dimethylsulfoxide-saline (MEL75), or 150 mg/kg melatonin + 10% dimethylsulfoxide-saline (MEL150). As in Experiment 1, solutions were prepared a few minutes prior to administration and animals were given an intraperitoneal injection immediately before HBO exposure.

HBO Exposure and Behavior Observation

HBO exposure and behavior observation were the same as Experiment 1. In this case, however, behavioral data were recorded electronically and later transferred to text for off-line analysis (see Appendix B for example text file).

RESULTS

The difference between seizure onset time and the time at which the animal reached 6 ATA was calculated to measure seizure latency to the nearest minute. Mean seizure latencies were calculated for each treatment; DMSO+saline (37.1, SD = 22.5), MEL75 (33.5, SD = 19.0),

and MEL150 (25.5, SD = 11.3). Two animals were surfaced for pulmonary reasons, one in the DMSO+saline group (latency = 84 min), and one in the MEL75 group (latency = 79 min). These animals elevated their respective treatment mean and standard deviation, but had no bearing on the final statistical outcome. There were no significant differences among treatment pairs (MEL75 vs. DMSO+saline, $t(18) = -.39, p > .05$; MEL150 vs. DMSO+saline, $t(18) = -1.46, p > .05$; MEL150 vs. MEL75, $t(18) = -.1.14, p > .05$).

CONCLUSIONS

These results suggest that melatonin pretreatment at the dosages used provides no benefit against the occurrence of HBO-induced seizure. We did not observe any effect of intraperitoneal administration of melatonin on HBO-induced seizure latency. Melatonin doses employed here (10, 75 and 150 mg/kg) were comparable to doses producing prophylactic effects in other animal seizure models. Although statistical power was less than anticipated in Experiment 2 given the longer mean seizure latency in the control condition (DMSO+saline) and slightly elevated standard deviations, mean seizure latencies in all of the melatonin conditions were not different from anticipated latencies with no intervention.

Perhaps the severity of this profile (6 ATA HBO) overwhelmed assumed antioxidant defenses of melatonin. Previous research on melatonin's capacity to reduce oxidative stress was conducted under less toxic conditions (4 ATA HBO)(4). It is unclear whether melatonin would limit CNS O_2 toxicity in a less severe hyperbaric environment.

RECOMMENDATIONS

1. Application of melatonin to prolong or eradicate the onset of HBO toxicity (seizures) in human diving investigations is not recommended at this time.

2. Further nonhuman animal research on the prophylactic capability of melatonin in an acute HBO environment is not recommended at this time. However, additional studies could be conducted at NMRC with intracerebroventricular administration to elucidate how this method produced the most dramatic anticonvulsant effects of melatonin in other seizure models (17).

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APPENDIX A: DATA SHEET

HBO DIVE DATA

SUBJECT INFO

DATE: _____
RAT: _____
WT(g): _____
SURG DATE: _____
INJ TIME: _____
DRUG:

PREDIVE OBSERVATION
Initials: _____
Time Behavior

DIVE PROFILE

SF: _____
LS: _____
RB: _____
DEPTH: _____
LB: _____
RS: _____

DIVE OBSERVATION
Initials: _____
Time Behavior

The dive observation format continued on the back side.

CH-chewing, **DG**-digging/sifting,
DR-drinking, **E**-eating,
EF-ear flick, **EX**-exploration,
FC-facial clonus,
FLC-forelimb clonus, **G**-grooming,
GTC-generalized tonic-clonic seizure, **HB**-heavy breathing,
HJ-head jerk, **HN**-head nodding,
HR-head rearing, **HS**-head shake,
J-jerks (body),
LOB-loss of balance,
OV-obstructed view,
PS-pulmonary stress, **Q**-quiet,
R-rearing (body),
RS-running seizure, **S**-still,
SN-sniffing, **TC**-tail clonus,
TE-tail extension,
TFE-tonic forelimb extension,
THE-tonic hindlimb extension,
TW-twitching, **WDS**-wet dog shake

SEIZURE LATENCY

APPENDIX B: EXAMPLE OF TEXT DATA FILE

Annotations in brackets are provided to clarify code scheme and were not part of the original data file.

MEL47 365g Friday, March 17, 2000
11:21:51 AM 75 mg/kg melatonin + DMSO IP
11:26:12 AM SF [start flush]
11:27:42 AM LS [leave surface]
11:31:10 AM RB 6 ATA 100% O2 [reach bottom]
11:31:18 AM Q [quiet—no noteworthy activity or end of previously noted activity]
11:33:02 AM head movement
11:33:26 AM change position
11:34:21 AM lethargic head movement
11:34:37 AM change position
11:34:53 AM brief SN [sniffing]
11:35:06 AM change position
11:35:25 AM change position
11:37:37 AM occasionally moves head, eyes open, head elevated, calm
11:38:49 AM change position
11:39:03 AM change position
11:40:16 AM occasional head movement
11:47:43 AM change position
11:48:47 AM periodic head movement
11:49:10 AM brief SN
11:49:22 AM change position
11:49:25 AM sniffing
11:49:48 AM Q
11:50:02 AM change position
11:50:15 AM sniffing
11:50:26 AM exploring cage
11:50:54 AM Q
11:51:09 AM jerk
11:51:19 AM twitching
11:52:59 AM jerk
11:53:25 AM jerk
11:53:56 AM jerk
11:54:14 AM jerk
11:54:34 AM jerk
11:55:29 AM sniffing
11:55:38 AM change position
11:55:42 AM jerk
11:55:49 AM jerk
11:55:51 AM jerk
11:56:00 AM facial clonus [onset of first hallmark behavior]
11:56:07 AM forelimb clonus
11:56:13 AM ear flicks
11:56:33 AM Q [end of hallmark behaviors greater than 20 s duration, ∴ seizure latency = 25 min]
11:57:02 AM end observation
11:57:05 AM LB [leave bottom]
11:58:10 AM RS [reach surface]